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Cytotoxic T Lymphocyte Responses of Infants After Natural Infection or Immunization With Live Cold-Recombinant or Inactivated Influenza A Virus Vaccine

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The cytotoxic T lymphocyte (CTL) response of infants after immunization with either inactivated trivalent subvirion vaccine (TIV) or bivalent attenuated cold-recombinant (CR) vaccine or occurrence of natural influenza virus infection were compared in a blinded, placebo-controlled study during the 1987–1988 and 1988–1989 influenza epidemic seasons. Healthy infants between 6 and 13 months of age were randomly assigned and administered a single dose of intranasal bivalent (A/H3N2/A/H1N1) CR vaccine, a two-dose regimen of TIV (A/H3N2/A/H1N1/B) influenza vaccine, or placebo. Peripheral blood lymphocytes were obtained prior to and 2–8 weeks after vaccination and at the end of the epidemic season and stimulated with virus in vitro for 6 or 7 days. Lysis of autologous virus-infected target cells was assessed in a 4 hr ^{51}Cr release assay. MHC class I-restricted influenza A-specific CTL was stimulated following natural influenza A virus infection but not after immunization with CR influenza A virus vaccine or TIV. These results demonstrate for the first time induction of influenza virus-specific CTL activity in infants under 1 year of age.

KEY WORDS: cell-mediated immunity, children, viral vaccines

INTRODUCTION

Cytotoxic T lymphocytes (CTL) appear to be important for recovery from influenza virus infection [McMichael et al., 1983; Ada and Jones, 1986; Ada et al., 1981]. Live attenuated and inactivated influenza virus vaccines have been shown to boost CTL responses in normal adults, and the kinetics of the responses have been described [Ennis et al., 1981; McMichael et al., 1981]. These vaccinations almost certainly represent secondary stimulation in subjects whose primary immunization to influenza virus occurred earlier through natural infection. The occurrence of high frequencies of infection with influenza viruses among infants and children has been well documented [Glezen et al., 1983, 1984; Wright et al., 1977].

Recent studies have evaluated safety and antibody responses to live attenuated cold recombinant (CR) virus influenza vaccines in infants and young children [Johnson et al., 1986; Feldman et al., 1985; Anderson et al., 1989]. The nature of CTL responses in these children for whom vaccination may represent primary exposure to influenza virus has not been described.

Investigations of cell-mediated immunologic (CMI) responses to viruses have often employed only in vitro lymphoproliferative responses of peripheral blood lymphocytes (PBL) to the virus. Lymphoproliferative responses of PBL to influenza virus have not always correlated with serum antibody titers to virus [Ruben and Bachmayer, 1978; Zahradnik et al., 1983; Dolin et al., 1978; Lazar and Wright, 1980]. Moreover, there is uncertainty in the interpretation of significance of lymphoproliferative responses to influenza virus when they are the sole measure of T-cell response. The purpose of this study was to assess CTL response of infants after primary immunization with inactivated trivalent subvirion vaccine (TIV) given intramuscularly (IM), bivalent live attenuated cold-adapted recombinant (CR) vaccine administered intranasally (IN), or occurrence of natural infection.

MATERIALS AND METHODS

Vaccines

Bivalent CR influenza A virus vaccine consisted of A/Bethesda/1/85-CR90 (H3N2) virus and A/Kawasaki/

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9/86-CR125 (H1N1) virus for the 1987–1988 epidemic season; A/Los Angeles/2/87-CR149 (H3N2) was substituted for A/Bethesda/1/85-CR90 for the 1988–1989 season [Piedra et al., 1993]. Each virus was prepared by reassortment between wild virus containing the specified hemagglutinin (H) and neuraminidase (N) surface glycoproteins and the internal proteins of A/Ann Arbor/6/60 (H3N2) that was adapted for growth at 25°C. Each virus was present in the vaccine in a final titer of $10^{6.5}$/50% tissue culture infective dose ($TCID_{50}$) per 0.5 ml. Subvirion inactivated trivalent influenza virus vaccine (TIV) consisted of A/Leningrad/360/86 (H3N2) A/Taiwan/1/86 (H1N1) and B/Ann Arbor/1/86 viruses for the 1987–1988 season; in the 1988–1989 season, A/Sichuan/2/87 (H3N2), A/Taiwan/1/86 (H1N1), and B/Victoria/2/87 viruses were used. The TIV contained 15 μg hemagglutinin (HA) of each viral component per 0.5 ml.

Study Populations

Thirteen healthy infants between 9 and 13 months of age (10.9 ± 1.6 months) were enrolled in the 1987–1988 study, and 29 infants between 6 and 13 months of age (6.5 ± 1.3 months) were enrolled in the 1988–1989 study from families participating in the Houston Longitudinal Family Study or receiving health care in one of two local clinics. None of the infants had a history of previous natural influenza virus infection or immunization [Piedra et al., 1993]. Healthy adults between the ages of 25 and 50 years were recruited at various times during the study, and their PBL were used for the purposes of standardizing the CTL assay and to serve as positive controls.

Study Design

Infants were assigned randomly to receive CR IN, TIV IM, or placebo (sterile saline). The IN group received a single 0.5 ml dose of sterile saline (PL_{IN}) or bivalent CR vaccine ($10^{6.5}$ $TCID_{50}$ of each component). The IM group was given 0.25 ml sterile saline (PL_{IM}) or TIV (7.5 μg HA per virus) at the time of enrollment and 4 weeks later. Infants in the IN group were evaluated for 2 weeks following inoculation for illness and shedding of virus (in nasal washes); those in the IM group were followed for reactions for 7 days after each injection for a total of 14 days. Blood samples were obtained from infants at enrollment (prevaccination), 2–4 weeks (for only the 1987–1988 season), 6–8 weeks after vaccination (postvaccination), and at the end of the influenza epidemic season (postseason).

Human Blood Processing

Peripheral blood was collected in preservative-free heparinized Vacutainer-evacuated tubes. PBL were separated on LeucoPrep (Becton Dickinson, Mountain View, CA) by centrifugation at 1,800*g* for 15 min. Plasma was removed and stored at −20°C for serological tests. Recovered PBL (1–5 × 10^6/ml blood) were washed twice. In some instances, aliquots of cells were placed in cryopreservation medium consisting of minimal essential medium plus 10% dimethyl sulfoxide (DMSO) and 10% fetal bovine serum (FBS) and then stored in a −70°C Revco freezer or in the gaseous phase of liquid nitrogen (−196°C) in a storage tank. Frozen cells were recovered by a rapid thaw in a 37°C water bath, transferred to cell culture medium, and washed twice. Cell viability was determined by trypan blue dye exclusion.

Viruses Used for CTL and Antibody Assays

Influenza virus A/Taiwan/1/86 (H1N1), A/Mississippi/1/85 (H3N2), A/Leningrad/360/86 (H3N2), B/Victoria/2/87, and B/USSR/1/86 were propagated in the allantoic cavity of 10-day-old embryonated eggs. After 3–4 days of incubation, the allantoic fluid was havested, clarified by centrifugation at 1,800*g* and stored in aliquots at −70°C. The viruses were further passaged in Mardin-Darby canine kidney tissue cultures (MDCK), and a 50% tissue culture infectious dose ($TCID_{50}$) was determined as previously described [Frank et al., 1979; Mbawuike et al., 1990].

Induction of In Vitro CTL Reponses

CTL reponses to influenza viruses were generated in six-well tissue culture plates (Costar, Cambridge, MA) in medium consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10^{-5} M 2-mercaptoethanol (2-ME), 10 mM HEPES buffer, and 10% heat-inactivated (56°C, 30 min) FBS as prevously described [McMichael et al., 1978; Mbawuike et al., 1993]. Briefly, stimulator (S) cells were prepared by incubating 4 × 10^6 PBL with 100 μl of A/Taiwan/1/86, A/HK/68, or B/USSR virus at 37°C in a 5% CO_2 humidified air incubator for 2 hr, with rocking. The cells were washed twice and cocultured with autologous responder (R) cells (2 × 10^7) at an S:R ratio of 1:5 and a final cell concentration of 2 × 10^6/ml for 7 days. Effector (E) cells so induced were washed once and resuspended in assay medium at 2.5 × 10^6/ml.

^{51}Cr Release Assay for CTL

On the day before assay, frozen cells to be used for targets were thawed quickly in a 37°C water bath, washed twice, and incubated in 10% FBS RPMI 1640 medium at 37°C overnight to allow them to recover from freezing. The cells recovered (50–100%) were >90% viable by trypan blue dye exclusion. On the day of assay, cells were pelleted by centrifugation at 400*g* for 5 min and resuspended in 100 μl A/Taiwan, A/HK/68, or B/USSR virus in the presence 100 μCi ^{51}Cr (Na_2CrO_4, specific activity 400–1,200 Ci/mM; DuPont NEN Research Products, Boston, MA) and incubated at 37°C, with rocking. After 2 hr the cells were washed, counted, and incubated in assay medium (RPMI 1640 plus 2 mM/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 mM nonessential amino acids, and 5% FBS) and washed. Effector cells (0.1 ml) and constant numbers of ^{51}Cr-labelled virus-infected target cells (5 × 10^3 or 1 × 10^4 in 0.1 ml) were placed at varying E:T ratios in quadruplicate in round-bottomed microtiter wells (Corning, Corning, NY) and centrifuged at 100*g* for 3 min. The cell mixtures were then incubated at

37°C for 4 hr; aliquots of supernatants were harvested following centrifugation at 450*g* for 15 min at 4°C. Gamma emissions in these fluids were counted to determine ^{51}Cr release. Results, expressed as percentage lysis, were calculated from the following formula:

$$\frac{(\text{Experimental release} - \text{Spontaneous release})}{(\text{Maximal release} - \text{Spontaneous release})} \times 100.$$

Spontaneous and maximal release consisted of targets incubated with medium alone and 1% Triton X-100, respectively, and ranged from 10% to 25%. Virus-specific lysis was calculated as a difference between virus-stimulated and unstimulated effector cells. A percentage lysis ≥10% was considered positive and was confirmed by a Students t test [McMichael and Askonas, 1978; Mbawuike et al., 1993].

Virus Isolation and Neutralizing Antibody (Nt Ab) Test

Methods for isolation and identification of human respiratory viruses have been described previously from this laboratory [Frank et al., 1979]. Serum Nt Ab activity determinations to influenza viruses were performed in microtiter plates using MDCK cells as previously described [Piedra et al., 1993]. A fourfold or greater rise in Nt Ab was considered significant. An influenza virus infection was considered to have occurred if the Nt Ab titer increase was significant and/or influenza type A was detected in the nasal wash specimen.

Statistical Analyses

Student's t tests, ANOVA, and Fisher's exact test were performed using True Epistat statistical software (Epistat Services, Richardson, TX). Comparisons of geometric mean antibody titers (GMT) between groups were made by one-way ANOVA and the Newman-Keuls multiple comparison test.

RESULTS

CTL Responses in Normal Adults

Prior to initiating the infant vaccine studies, we characterized influenza virus-specific CTL responses in adults for specificity, MHC restriction, cellular requirements, and effector cell phenotype to ensure our ability to develop data comparable to those previously reported [McMichael and Askonas, 1978; Ennis et al., 1981; Mbawuike et al., 1993]. Results obtained from adult donors assayed at different times over a 1 year period using influenza A (H1N1 and H3N2) and influenza B viruses showed that CTL effector cells were cross-reactive for influenza A (H1N1 and H3N2), but not for influenza B, and that lytic activity was mediated by $CD3^+$, $CD8^+$, $CD4^-$ cells (data not shown). To ensure that cryopreserved lymphocytes were functional, fresh and frozen cells from three adults were evaluated for CTL generation. Following stimulation with influenza B/USSR, A/HK/68, or A/Taiwan, lymphocytes frozen for 2–4 weeks exhibited CTL activity comparable to that of fresh cells (data not shown).

CTL and Antibody Responses of Infants: 1987–1988 Season

Details of clinical, serological, and virological response data have been previously published [Piedra et al., 1993]. Table I presents the viral shedding and antibody response data following influenza vaccination. All CR recipients shed influenza A (H1N1) virus for 2 days or more after inoculation, whereas only four of six shed H3N2 virus for 1 day each. Among the IM group, one placebo and one TIV recipient shed H3N2 and H1N1 virus, respectively, during the influenza season. Table I also shows that five of six CR recipients developed a fourfold or greater rise in Nt Ab antibody titer to influenza A/Taiwan (H1N1), and one of these recipients also developed a rise to A/Mississippi (H3N2) virus, 6 weeks following vaccination. By the end of the influenza epidemic season, all CR recipients had serological evidence of H1N1 infection, and four of six showed evidence of H3N2 infection. Because no influenza virus infections were detected by culture, seroconversion at the end of the epidemic season may be the result of delayed responses. One of the two TIV recipients exhibited an Nt Ab rise to influenza A/H3N2 and A/H1N1 as well as to influenza B. None of the placebo recipients had a rise, except for infant 10, who may have experienced a natural infection with influenza A/H3N2 prior to vaccination. Similar patterns of influenza hemagglutination inhibition antibody (HAI) responses were observed (data not shown).

Table II shows individual specific CTL activity inducible by in vitro restimulation with A/Taiwan virus and lytic activity against A/Taiwan-infected autologous target cells in PBL of infants in the 1987–1988 study. Eleven of thirteen infants tested lacked inducible CTL activity to A/Taiwan before vaccination. Inducible CTL activity to influenza B virus (B/USSR) was not detected (data not shown). Data for 2–4 and 6–8 weeks following vaccination were incomplete owing to loss of cells with cryopreservation. However, data for five of the six CR influenza A virus recipients show that two had developed inducible CTL activity (>10% specific lysis) to influenza A virus by 2–4 and 6–8 weeks following vaccination. One of the initially negative placebo recipients (No. 10) developed inducible CTL activity at 6–8 weeks. This infant had developed serological evidence of an H3N2 infection by 6 weeks after receiving a placebo vaccination; sporadic infections were detected in the community at this time. The apparent infection would account for acquisition of the positive CTL response. Thus, there was no difference in CTL resposnes between CR vaccines compared to placebo and TIV recipients (3/5 vs. 1/7) at the postvaccination period.

Following the epidemic season (20–32 weeks after vaccination), lymphocytes were obtained, stimulated with A/Taiwan virus, and assayed for lysis of autologous cells infected with A/Taiwain or B/USSR (for specificity) or A/Taiwan-infected nonautologous cells (HLA restriction). Nine of thirteen infants tested exhibited inducible CTL activity (Table II). All but three of the 13 infants

(Nos. 8, 9, and 11) exhibited evidence for influenza A virus infection over the epidemic season as indicated by a positive culture for an influenza A virus (Nos. 10 and 13) and/or a fourfold or greater antibody response [Piedra et al., 1993; data not shown]. CTL activities for two of the three lacking such evidence were either negative or low (12%), whereas PBL from the other infant (No. 11) exhibited the highest CTL activity detected. Although the latter infant had no laboratory evidence of influenza virus infection, his 3-year-old sister had culturally and serologically documented influenza A virus infection. Uniformity for the assay procedure was demonstrated by inducible CTL activity in a normal adult at each time period.

The specificity of A/Taiwan-induced CTL activity was evaluated. A majority of A/Taiwan-induced effector cells lysed A/Taiwan-infected autologous target cells (9 of 11) but not B/USSR-infected autologous target cells (Table II). Two infants exhibited CTL activity against B/USSR-infected autologous cells but at a lower level than that for A/Taiwan-infected cells (14% and 26% vs. 26% and 44%, respectively). Nonspecific CTL responses have previously been attributed to NK-like T cells generated in the stimulation with influenza virus [McMichael et al., 1982]. CTL responses appeared to be HLA-restricted; nonautologous target cells infected with A/Taiwan were not lysed. Even though HLA typing was not performed on these cells, the CTL data suggest HLA dissimilarity.

CTL Responses of Infants: 1988–1989 Season

Lymphocytes were stimulated with influenza A/Taiwan-infected autologous cells and tested for lysis of autologous target cells infected with A/Taiwan or B/USSR virus or nonautologous A/Taiwan-infected target cells. A summary of influenza A/Taiwan-virus-specific CTL and antibody responsiveness for the 26 infants studied during the 1988–1989 season is presented in Table III. Data for three infants were not included owing to inadequate cell recovery. Because of loss of cells with cryopreservation the previous year, all cells were tested when fresh. Two of twenty-six infants tested exhibited low (12% and 14%) CTL activity against A/Taiwan virus before vaccination. The CTL responses in the two prevaccine samples and adults were specific for influenza A/Taiwan and were allorestricted (data not shown). At 6–8 weeks following vaccination (postvaccination), none of 26 infants tested had CTL activity. The CTL responses of the two who were initially positive had declined to lower than 10%.

Most of the 26 infants had detectable neutralizing antibody to A/H3N2 prior to vaccination [Piedra et al., 1993]. The antibodies were assumed to have been passively acquired from mothers [Griffiths et al., 1982], because influenza A/Los Angeles (H3N2) was the predominant virus that circulated in the community in the year prior to study initiation. Following vaccination, eight of nine CR recipients shed H3N2 CR virus after vaccination, whereas none shed H1N1; TIV and placebo recipients also did not shed virus. All CR infants developed an antibody response to H3N2 virus, whereas only two developed a rise to the H1N1 [Piedra et al., 1993].

TABLE I. Influenza Virus Infection and Neutralizing Antibody Responses in Infants Who Received Bivalent CR or TIV Vaccine

Number	Vaccine[a]	CR vaccine virus shedding[b]		Serum Nt Ab titer (log_2) to:[c]							
				A/Mississippi (H3N2)			A/Taiwan (H1N1)			B/Ann Arbor	
		H3N2	H1N1	Prevaccine	Postvaccine	Postflu season	Prevaccine	Postvaccine	Postflu season	Prevaccine	Postvaccine	Postflu season
1	CR	+	+	0.5	0.5	3.5	0.5	1.5	3.5	0.5	0.5	0.5
2	CR	+	+	2.0	3.0	3.5	0.5	4.5	6	0.5	0.5	0.5
3	CR	−	+	1.5	1.5	6	0.5	3.5	4.5	0.5	0.5	0.5
4	CR	+	+	1.5	4.0	5.5	1.0	3.5	3.5	0.5	0.5	0.5
5	CR	−	+	1.5	2.5	6.0	1.5	4.0	4.5	0.5	0.5	0.5
6	CR	+	+	0.5	0.5	0.5	0.5	7.0	8.5	0.5	0.5	1.5
7	PL_{IN}	−	−	3.5	2.5	4.0	5.0	4.5	3.5	0.5	1.0	0.5
8	PL_{IN}	−	−	1.0	1.5	1.5	0.5	0.5	0.5	0.5	0.5	1.0
9	PL_{IM}	−	−	2.5	2.0	2.5	1.5	0.5	0.5	0.5	0.5	0.5
10	PL_{IM}	+[d]	−	0.5	0.5	5.0	0.5	0.5	1.0	0.5	0.5	0.5
11	PL_{IM}	−	−	2.5	1.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
12	TIV	−	−	3.0	5.0	4.0	0.5	5.0	3.5	0.5	3.0	1.5
13	TIV	−	+[d]	2.0	3.5	3.5	2.0	3.0	4.0	0.5	1.5	0.5

[a]CR, bivalent cold-recombinant live-attenuated vaccine containing A/Kawasaki (H1N1-CR 125) and A/Bethesda (H3N2-CR 90); Pl, placebo; IN, intranasal; IM, intramuscular; TIV, trivalent inactivated virus vaccine containing antigens of influenza A/Taiwan (H1N1), A/Leningrad (H3N2), and B/Ann Arbor viruses.
[b]Infants who received IN drops were cultured every other day for 14 days after IN application. Infants who received IM shots were monitored by phone every other day for 1 week after each IM shot (for a total of 14 days) and were cultured for virus only if they were seen by a physician. CR vaccine recipients shed influenza A/H1N1 or A/H3N2 virus 3–10 days postvaccination.
[c]Serum samples were obtained prior to vaccination (prevaccine), 2–4 and 6–8 weeks following vaccination (postvaccine), and at the end of the influenza epidemic period (postflu season). Antigens used for NT Ab tests included A/Taiwan/1/86 (H1N1), A/Mississippi/1/85 (H3N2), and B/Ann Arbor/1/86. An antibody response is represented by a fourfold or greater increase in neutralizing antibody titer to A/H1N1 or A/H3N2 or both.
[d]Wild-type virus isolated over the epidemic season.

TABLE II. Influenza Virus-Specific Cytotoxic T Lymphocyte Response of Infants Given Live or Inactivated Influenza Virus Vaccines*

Number	Vaccine	Autologous A/Taiwan (Prevaccine)	Percentage total lysis of[a]: Autologous A/Taiwan		Autologous A/Taiwan (postepidemic period)	Autologous B/USSR (postepidemic period)	Nonautologous A/Taiwan (postepidemic period)
			Postvaccine (2–4 weeks)	Postvaccine (6–8 weeks)			
1	CR	0	6	14	13	8	NT
2	CR	0	NT	NT	5	NT	1
3	CR	NT[a]	1	<1	26	14	0
4	CR	0	0	0	44	26	0
5	CR	0	23	NT	17	2	0
6	CR	0	14	5	9	7	0
7	Pl_{IN}	6	8	NT	21	0	0
8	Pl_{IN}	0	NT	0	12	3	2
9	Pl_{IM}	NT	NT	NT	9	NT	NT
10	Pl_{IM}	0	NT	15	15	0	0
11	Pl_{IM}	0	9	3	55	8	4
12	TIV	0	NT	0	7	1	2
13	TIV	5	0	NT	17	0	0
Adult[b]	None	27	41	30	21	0	5

*PBL were stimulated with autologous cells infected with A/Taiwan (H1N1) virus at a stimulator:responder ratio of 1:20 for 7 days. Effector cells so generated were incubated with A/Taiwan or B/USSR virus-infected autologous or A/Taiwan virus-infected nonautologous target cells at an E:T ratio of 20:1. Values represent total percentage lysis of virus-infected target cells.
[a]NT, not tested.
[b]Adult volunteer with no recent vaccination or clinical infection.

TABLE III. Frequency of Influenza Virus-Specific Cytotoxic Lymphocyte and Antibody Responses Among Vaccinated Infants During 1988–1989

Vaccine group[a]	N	Prevaccination (No. with CTL[b])	Postvaccination period[c]			Postepidemic period	
			No. who shed vaccine virus	No. with Ab rise	No. with CTL	No. with influenza A infection[d]	No. with CTL[e]
CR	9	2/9	8/9	9/9	0/9	2/9	0/9
TIV	10	0/10	0/10	10/10	0/10	0/10	0/10
PL	7	0/7	0/7	2/7[c]	0/7	0/7	2/7
Adult[f]	2-3	2/2	NT	NT	2/2	NT	3/3

[a]CR, bivalent cold-recombinant live vaccine containing A/Kawasaki (H1N1-CR 125) and A/Los Angeles (H3N2-CR 149); Pl, placebo; TIV, trivalent inactivated virus vaccine containing antigens of influenza A/Taiwan (H1N1), A/Sichuan (H3N2), and B/Victoria viruses.
[b]Number of infants/number in group with ≥10% lysis of A/Taiwan-infected autologous target cells (12% and 14% CTL lysis). One of these infants experienced natural infection 2 weeks before the vaccination.
[c]Tested only at 6–8 weeks postimmunization.
[d]Ab rise to A/H1N1 over epidemic period and/or virus isolation.
[e]Frequency of CTL lysis of A/Taiwan-infected autologous target cells (12% and 16%).
[f]All three adult controls with no recent vaccination or clinical infection had CTL lysis of 14–31% when tested at various times during study.

All TIV recipients developed antibody to H1N1 virus, and three also responded to the H3N2 virus. Two of seven in the placebo group also developed antiinfluenza A antibody. At the end of the influenza epidemic season (postseason), none of the 26 infants exhibited CTL activity. Unlike the case in the previous season, influenza B virus was the predominant virus causing infection, although some type A virus was detected in the community.

Two of the CR vaccinees developed an antibody response over the epidemic period to A/Taiwan (H1N1) virus, but, as indicated, neither infant developed CTL activity. Normal adult controls exhibited inducible CTL activity to A/Taiwan at the three time points (14–31% lysis), indicating continued uniformity of the assay procedure.

DISCUSSION

These studies have demonstrated induction of influenza A virus-specific CTL activity in infants under 1 year of age. The 1987–1988 study showed that natural influenza A virus infection can induce CTL responses in infants whether or not they had been previously primed with influenza vaccine. Vaccination with attenuated bivalent CR influenza A virus vaccine might induce a response. Some positive CTL responses at the end of the influenza epidemic season could be due to undetected natural infection. Discordance between CTL and antibody responses to presumed infection might be due to differing kinetics but suggests that CTL can develop without measurable HAI and neutralizing antibody. Previous studies have demonstrated such inductions of CTL

in influenza vaccinees without measurable antibody responses [McMichael et al., 1983; Ennis et al., 1982].

The major conclusion from the results obtained from the second study year are in agreement with those of the previous year; i.e., vaccination with attenuated CR vaccine does not invariably induce CTL responses in infants. Even though the postvaccination CTL data (2–8 weeks) in the 1987–1988 study was inconclusive owing to loss of cryopreserved cells, the postvaccination data from 1988–1989 study strongly support the conclusion that none of the vaccines induced CTL responses in the infants and children. Additional studies are required to determine whether this phenomenon is due to a superiority of natural influenza over CR infection in inducing CTL responses, as has been suggested from some animal studies [Webster and Askonas, 1980]. It is possible that it was too late to detect CTL at 6–8 weeks; previous studies have shown that peak CTL responses occur in both adult humans and mice approximately 2–4 weeks after vaccination or infection [Ennis et al., 1981; McMichael et al., 1981]. A kinetic study of CTL induction will clarify this question (if parental objection to frequent blood draws from infants can be overcome). Positive CTL activity observed in 2 of 26 infants prior to vaccination (1988–1989 study) cannot easily be explained because of lack of evidence of natural influenza infection even though all infants had significant Nt Ab titer to H3N2 virus. Such antibody was presumably derived transplacentally, because H3N2 was a predominant epidemic virus in 1987–1988. However, the CTL activity present prior to vaccination declined to below 10% by 6–8 weeks after vaccination, suggesting a normal decay of activity and indicative of active immunity in the infants.

Unlike the case in the 1987–1988 season, when every infant had positive CTL responses for type A influenza at the end of the influenza epidemic season, only 2 of 26 tested had activity during the 1988–1989 study. This is thought to be due to the fact that the predominant epidemic virus in 1988–1989 was an influenza B virus, whereas H3N2 was predominant during the previous year. This is supported by the finding that a majority of the 1987–1988 study group had either virological or serological evidence of influenza A virus infection at the end of the season, and this did not occur the subsequent year.

The methods of CTL induction and target cell lysis of McMichael and Askonas [1978] were followed in the present study, and the levels of CTL obtained were comparable to published results [McMichael and Askonas, 1978; McMichael et al., 1981, 1982, 1983]. Phytohemagglutinin-stimulated and Epstein-Barr virus (EBV)-transformed cells when used as target cells give higher levels of CTL lysis. They also give high spontaneous release of ^{51}Cr and high CTL background lysis of uninfected or control targets. Thus, we routinely prepare CTL targets using cryopreserved PBL cultured overnight to recover before virus infection [Mbawuike et al., 1993] as described by McMichael and coworkers [1978, 1981, 1982, 1983]. In the present study, the viability of infant cells recovered after cryopreservation was high (81.2 ± 3.3%; range 54–95%) and was only slightly lower than that of young adults and, therefore, should have functioned well for target cell preparation.

This study highlighted the difficulties encountered in performing CTL studies in infants and children, such as low cell recoveries after culture or following cryopreservation. There was also the difficulty of interpreting the vaccine responses resulting from preexisting maternal antibodies [Griffiths et al., 1982]. Every effort was therefore made to ensure the validity of the CTL data obtained, for example, by including adult controls. Owing to cell number limitations, T-cell subset fractionation, CTL specificity, and HLA restriction could be performed only using adult cells. The limited available data, however, suggest that CTL in adults and infants has similar characteristics [Isaacs et al., 1987]. Therefore, the data presented herein represent a necessary first step in our understanding of the role of $CD8^+$ CTL activity in the control of influenza infection in infants and children.

In conclusion, we have demonstrated for the first time induction of influenza A-virus-specific CTL response in infants 6 months to 2 years of age. Vaccination with CR or TIV does not invariably induce detectable CTL activity in these infants, although natural influenza virus infection may do so. It is possible that multiple infection with CR vaccine can induce CTL, as indicated by the inability of one dose to induce antibody response in some individuals. The roles of age and number of exposures to natural influenza infection (or CR vaccinations) should be further assessed.

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